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PRIMER

Model systems for regeneration: zebrafish

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ABSTRACT

Tissue damage can resolve completely through healing and regeneration, or can produce permanent scarring and loss of function. The response to tissue damage varies across tissues and between species. Determining the natural mechanisms behind regeneration in model organisms that regenerate well can help us develop strategies for tissue recovery in species with poor regenerative capacity (such as humans). The zebrafish (*Danio rerio*) is one of the most accessible vertebrate models to study regeneration. In this Primer, we highlight the tools available to study regeneration in the zebrafish, provide an overview of the mechanisms underlying regeneration in this system and discuss future perspectives for the field.

KEY WORDS: Regeneration, Zebrafish, Cell progenitors, Injury methods, Genetic tools

Introduction

Most vertebrate tissues undergo continuous cell turnover to maintain tissue homeostasis, and cells that no longer exert their function correctly are eliminated and replaced by new ones. This continuous cellular turnover is not homogenous in all tissues: whereas gut enterocytes and skin keratinocytes show a high turnover (Young and Heath, 2000), cardiomyocytes (Bergmann et al., 2009; Senyo et al., 2013) and neurons are rarely replaced throughout adult life. In the case of neurons, proliferation is limited to specific regions in the brain (Ernst et al., 2014; Lim and Alvarez-Buylla, 2016). Beyond homeostatic tissue maintenance, some tissues and organs are capable of reacting to an injury by replacing the lesioned region. In this scenario, responses can either involve tissue repair, where a fibrotic scar replaces the injury, or regeneration, where new cells proliferate and/or differentiate into the same cell type as the one that was damaged, thus reconstituting the tissue so that it can recover its functional activity. As a general outline, during injury response the damaged area (Fig. 1A,B) is initially infiltrated by immune cells (Fig. 1C), which clean the dead tissue and prevent infections. This is followed by the deposition of extracellular matrix (ECM) (Fig. 1D), which provides support and structure to the injured area. ECM deposition results in the development of a, sometimes permanent, fibrotic scar. Alternatively, the lesioned region can be replaced by new tissue (Fig. 1E) in a multi-step regenerative process that can involve dedifferentiation, proliferation and re-differentiation of existing cells (as discussed further below). In some cases, the regenerative phase occurs concomitant with the regression of the transient fibrotic tissue. Overall, the resulting regenerated tissue has the same morphological and physiological properties as the lost tissue, and is hence fully functional (Fig. 1F) (Hardy, 1989).

Vertebrates vary widely in their regenerative capacity. Whereas mammals usually have a more limited regenerative capacity – restricted to few tissues or organs (e.g. skin and liver) or a short time window after birth [e.g. the heart (Porrello et al., 2011)] – other vertebrates possess a higher degree of regenerative capacity: e.g. axolotls are capable of regenerating several organs, including the heart (Cano-Martinez et al., 2010) and the limbs (Simon and Tanaka, 2013). Among the vertebrate animal models available for regeneration studies, the zebrafish (*Danio rerio*) – a 2-5 cm long freshwater teleost – is one of the most widely used. Following their establishment as a genetically and experimentally accessible model system for studying development (Haffter et al., 1996; Streisinger et al., 1981), early regeneration studies showed that zebrafish, like many other fish, are able to fully regenerate many tissues and organs (Bernhardt et al., 1996; Johnson and Weston, 1995; Poss et al., 2002; Raya et al., 2003; Rowlerson et al., 1997; White et al., 1994). This pioneering research convinced the scientific community to consider this model organism for studying organ regeneration as well as development. Some of the tissues and organs most typically used in regeneration studies include the spinal cord (Becker et al., 1998), brain and cerebellum (Kroehne et al., 2011; Liu et al., 2004), retina (Vihtelic and Hyde, 2000), hair cells (Harris et al., 2003), heart (Poss et al., 2002; Raya et al., 2003), caudal fin (Nechiporuk and Keating, 2002; White et al., 1994), kidney (Reimschuessel, 2001), and liver (Burkhardt-Holm et al., 1999).

Regeneration studies have been greatly facilitated by the ever-increasing power of genetic tools, including transgenesis and genome editing to generate loss-of-function mutations. (Hwang et al., 2013; Kawakami, 2007; Moore et al., 2012; Stuart et al., 1988). In comparison with other highly regenerative animal models such as the axolotl, these were developed much earlier in zebrafish,

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Model systems for regeneration

This article is part of a series entitled 'Model systems for regeneration'. This series of articles aims to highlight key model systems and species that are currently being used to study tissue and organ regeneration. Each article provides background information about the phylogenetic position of the species, its life-cycle and habitat, the different organs and tissues that regenerate, and the experimental tools and techniques that are available for studying these organisms in a regenerative context. Importantly, these articles also give examples of how the study of these models has increased our understanding of regenerative mechanisms more broadly, and how some of the open questions in the field of regeneration may be answered using these organisms. To see the full collection as it grows, please visit: https://dev.biologists.org/collection/regeneration_models

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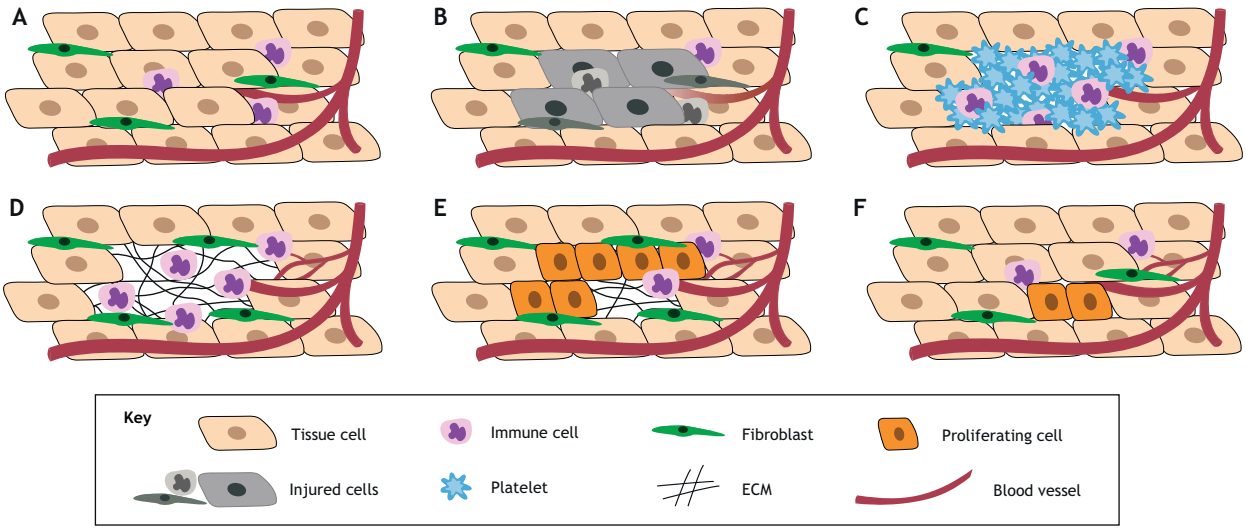

Fig. 1. Phases of tissue regeneration. (A) Uninjured tissue. (B) Injury: injured area is shaded in gray, representing damaged and dead cells. The blood vessel in direct contact with the lesion is also damaged and is marked in a lighter red color than the healthy vessels. (C) Wound closure and immune response: the injured area is infiltrated by platelets to stop the bleeding resulting from injury to the blood vessels in the damaged area. At the same time, the lesion is also infiltrated by immune cells. (D) Clearing of debris and deposition of extracellular matrix (ECM): the immune cells start to clean the dead tissue and other debris resulting from the injury. Fibroblasts proliferate and produce ECM fibers that fill the cleared injury area to prevent the injury from collapsing. The formation of new blood vessels takes place (thinner red lines). (E) Resorption of ECM and cell proliferation: the ECM starts to be eliminated as the injury is repopulated by new cells through proliferation. (F) Regeneration: the injured area has been completely repopulated and the tissue reconstituted to its homeostatic condition. Cells undergo cell cycle arrest and very few proliferating cells can be seen.

further stimulating its use for regeneration studies. Moreover, excellent platforms for sharing resources and knowledge (summarized in Table 1) are contributing to a steady growth of the research community working with this model organism, also in the context of regeneration. In this Primer, we provide a broad overview of the available tools for regeneration research in the zebrafish, and focus on the challenges and opportunities presented by this model organism.

Tools for studying regeneration in zebrafish

Many of the attributes that make zebrafish an appealing model for developmental biologists also give it significant advantages for regeneration researchers – with the genetic and genomic tools, and accessibility for live imaging and drug screening being particularly important. We discuss these topics further below, but focus first on the range of injury models available to researchers.

Tissue injury models

Several injury models have been established to study regeneration in zebrafish, including physical injury models such as resection, stabbing or cryoinjury, or, alternatively, genetic procedures to ablate a specific cell type (Table 2 and Fig. 2). Although most studies are performed in adult zebrafish, in some instances larvae are also used to study organ regeneration. Choosing the appropriate injury model, and developmental stage, is an important decision. First, one should take into account the type of injury intended to be mimicked – particularly if the primary aim is to provide a model for a human condition. However, it is not always possible to mimic a mammalian injury process. For example, myocardial infarction is caused by an ischemic event, often due to the occlusion of coronary vessels by an atherosclerotic plaque. This type of injury can be mimicked in mice, but is very challenging to recapitulate in the zebrafish, owing to its small size. Second, the choice of the injury method also depends on the biological question to be answered. To study global processes of regeneration, one probably needs to use a method that lesions the whole tissue, such as surgically induced injuries. If the aim is to understand the regenerative capacity of a specific cell type, then genetic ablation might be a better choice. As a further consideration, different types of injury can lead to changes in the efficiency of cell replacement or speed of regeneration, as

Table 1. Online resources for the zebrafish community

Resource	Description	URL and references
Zebrafish Information Network (ZFIN)	Database of multiple resources available for zebrafish studies, including protocols and available lines	http://www.zfin.org (Howe et al., 2013)
International Zebrafish Society (IZFS)	Promotion of zebrafish research, organization of conferences, travel fellowships and awards	https://www.izfs.org/
EUFishBiomed	Promotes exchange of information within the fish community, support of conferences and travel fellowships	https://www.eufishbiomed.kit.edu/ (Strahle et al., 2012)
Zebrafish International Resource Center (ZIRC)	Zebrafish stock center (USA) collecting existing zebrafish resources	https://zebrafish.org/
Karlsruhe Institute of Technology (KIT)	Zebrafish stock center (Europe) collecting existing zebrafish resources and providing training	https://www.ezrc.kit.edu/ (Geisler et al., 2016)
China Zebrafish Resource Center (CZRC)	China Zebrafish Resource Center collecting existing zebrafish resources, developing new lines and technology	http://en.zfish.cn/

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Table 2. Injury models for zebrafish organ regeneration

Organ	Injury method	References
Fin	Resection	Geraudie et al. (1993); Pfefferli and Jaźwińska (2015)
	Cryoinjury	Chassot et al. (2016)
Heart	Resection	Poss et al. (2002)
	Cryoinjury	Chablais et al. (2011); Gonzalez-Rosa et al. (2011); Schnabel et al. (2011)
	Genetic ablation	Wang et al. (2011)
Central nervous system (brain, spinal cord and eye)	Nerve crushing	Becker and Becker (2008); Bernhardt et al. (1996)
	Stabbing	März et al. (2011)
	Transection	Becker and Becker (2008); Becker et al. (1998)
	Visible light	Vihtelic and Hyde (2000)
	Heat	Raymond et al. (2006)
	Chemical	Fimbel et al. (2007)
Pancreas	Genetic ablation	Pisharath et al. (2007)
Liver	Resection	Sadler et al. (2007)
	Chemical	Cox et al. (2014)
Kidney	Chemical	Reimschuessel (2001)
Sensory hair cells	Chemical	Harris et al. (2003)
Skin	Laser	Richardson et al. (2013)

exemplified for the spinal cord (Ohnmacht et al., 2016) and the heart (Gonzalez-Rosa et al., 2011).

Surgical injuries

One of the most common surgical methods is resection (amputation) of the tissue. This technique is commonly used for fin and heart. Following fin amputation, the first step towards regeneration involves the development of an epidermal cap that closes the injury. Underneath, a blastema comprising undifferentiated cells is formed (discussed further below). After initial proliferation, these cells redifferentiate, culminating in the formation of new fin tissue (reviewed by Pfefferli and Jaźwińska, 2015). In the heart, the first step towards regeneration after ventricular resection is the formation of a blood clot, later replaced by a fibrin clot, followed by *de novo* growth to replace the lost tissue, until the damaged myocardium becomes almost indistinguishable from the surrounding tissue (reviewed by Gonzalez-Rosa et al., 2017).

Another popular surgically induced lesion method is cryoinjury, which is frequently used for the heart (Chablais et al., 2011; Gonzalez-Rosa et al., 2011; Schnabel et al., 2011) and has also been described in fin (Chassot et al., 2016). In this method, the tissue is exposed to a super-cooled metal, which effectively kills the cells it touches. In the fin, after the dead tissue falls off, regeneration proceeds as in the amputation model (Chassot et al., 2016). By contrast, the regenerative process in the heart is a little different: it begins with the replacement of the necrotic tissue with ECM-depositing fibroblasts. Subsequently, the transient scar tissue regresses and is replaced by new cardiac tissue (Gonzalez-Rosa et al., 2012). As well as low temperatures, cell death can be induced by exposure to high temperatures, either using a heated metal rod (Raymond et al., 2006) or by applying a light source in a determined region (Conedera et al., 2017; Vihtelic and Hyde, 2000).

Finally, another method is stabbing, commonly used to damage the central nervous system, including the spinal cord (Becker et al., 1998). In this case, accumulation and proliferation of glial cells in the damaged area is followed by the proliferation of progenitors that ultimately differentiate into new nervous tissue (März et al., 2011).

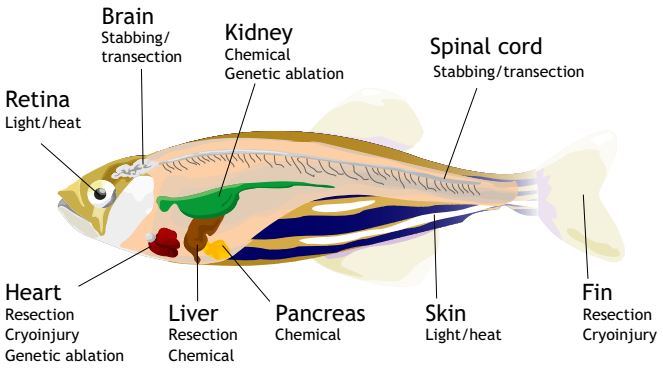

Fig. 2. Organ regeneration in the zebrafish. Summary of some of the organs and tissues used for regeneration studies in zebrafish. Preferred injury models are annotated for each organ.

Chemically induced cell damage

Besides physical stimuli, organ injuries can be induced by chemical compounds, which can either be directly administered to each individual fish by intraperitoneal injection, perorally or by dissolving in the fish water followed by ingestion. The toxicity of gentamicin is used to produce kidney or lateral line injuries (Kamei et al., 2015) and acetaminophen is used to induce liver injuries (Cox et al., 2014). After an initial phase of cell death, proliferation and cell migration lead to the repopulation of the damaged area, as has been demonstrated in studies on zebrafish kidney (Reimschuessel and Williams, 1995), lateral line hair cell (Harris et al., 2003) and liver (Burkhardt-Holm et al., 1999) regeneration. This method, however, presents some disadvantages, such as off-target effects, as well as a less uniform injuries caused by unequal drug distribution (Strahle and Grabher, 2010).

Genetic ablation

This approach allows tissue- or cell type-specific death and can also provide temporal control of cell ablation. In several transgenic zebrafish lines, cell death can be specifically induced in targeted cells. Methods include the regulated expression of diphtheria toxin A chain (Wang et al., 2011) and overexpression of a cytotoxic fluorescent protein, KillerRed – which has been used to ablate specific cell types through photoxicity upon intense light illumination (Del Bene et al., 2010). Perhaps the most widely used system is based on the ability of the

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Table 3. Genetic tools for zebrafish regeneration studies

Method	Application	Pros	Cons
Fluorescent reporter lines	Cell/tissue imaging	Allow evaluation of gene expression pattern by promoter/enhancer-driven fluorescent reporter expression	Detection limited to promoter activity, not suited for fate mapping Regulatory region might not fully recapitulate endogenous gene expression pattern Fluorescent protein expression might be more stable than the endogenous gene
Photoconvertible proteins (e.g. KAEDE)	Lineage tracing	Activated by light Produces a faster response than chemically induced systems	Difficult to apply in non-superficial adult tissues Transient labelling Leakiness
CreER^{T2}/loxP	Lineage tracing Gene overexpression	Temporal and spatial conditional gene expression Often used for lineage tracing studies	Cannot be turned off Requires chemical treatment Can be leaky (Cre activity even without tamoxifen induction)
Dre/rox	Lineage tracing Gene overexpression	Conditional gene expression If combined with CreER^{T2}/loxP, it allows binary recombination studies Dre can be linked to estrogen or progesterone receptor to make it inducible (as with Cre)	Cannot be turned off Not well established in the zebrafish Fewer lines available than for Cre/Lox
TetOn/TetOff	Gene overexpression	Specific gene visualization and/or overexpression in a spatio-temporally controlled manner System can be turned on and off as needed	Few available lines Doxycyclin toxicity not well assessed
Heat-shock promoter	Gene overexpression	Temperature-dependent inducible expression Does not require chemical treatment	Gene expression levels are temperature dependent: precise temperature settings are critical for the method to work Promotor not equally efficient in all cell types
Gal4/UAS	Gene overexpression Cell/tissue imaging	Spatially controlled gene expression	Transcriptional silencing when Gal4 is expressed from very active promotors: mostly limits its use to developmental stages
TALEN	Genome editing and generation of mutant lines	Highly specific and efficient More versatile for sequence recognition than CRISPR	Time consuming Has not been applied to knock-in approaches
CRISPR/Cas9	Genome editing and generation of mutant lines	sgRNA easier to produce than TALEN Targeting of various genomic regions with one injection	Off-target effect possible Use for site-directed insertions still not fully established

bacterial enzyme nitroreductase to convert the prodrug metronidazol into a cytotoxin, leading to the death of nitroreductase-expressing cells. This technique was first described in studies on pancreatic β-cell regeneration in zebrafish (Curado et al., 2007; Pisharath et al., 2007). Recently, a more potent prodrug, nifurpirinol, has been identified as a more efficient and specific compound (Bergemann et al., 2018). In all cases, genetically targeted cells undergo apoptosis. However, in contrast to the cryoinjury model, ECM deposition is limited (Wang et al., 2011). Following cell death, cells from the surrounding healthy tissue begin proliferating to replace the lost tissue.

Genetic tools

As in other fields of zebrafish research, the use of genetic tools is essential to understand the role played by different genes during regeneration. In Table 3 we summarize most of the genetic modification approaches discussed in the ensuing section and include the main advantages and disadvantages of each of them.

Transgenesis

There is a wide range of tools available for the generation of transgenic lines. The most commonly used include the use of Tol2 transposons (Kawakami et al., 2000) and I-SceI meganuclease (Thermes et al., 2002), which facilitate the integration of foreign DNA into the fish genome. Alternatively, BAC recombineering (Bussmann and Schulte-Merker, 2011; Suster et al., 2011) allows the insertion of larger fragments of DNA that often better recapitulate endogenous gene expression patterns. Of note, insertion into the genome is non-directed in these approaches. The lack of a ROSA26-like landing site has been a caveat; the use of Phi31 Integrase transgenesis (Mosimann et al., 2013) has been reported as a potential way to address this, and the problem will hopefully be solved using CRISPR/Cas9 approaches. Transgenic fluorescent reporter lines used to tag/track a particular cell type or report a gene expression pattern have proven to be essential for investigating regeneration.

Inducible gene expression and genetic fate mapping

Different techniques have been established that allow the use of genetic tools in a spatially and/or temporally controlled manner during regeneration.

Heat shock

Inducible gene expression can be achieved by using a heat-shock driven system (Ádám et al., 2000). The heat-shock promoter hsp70 is used to drive expression of the gene of interest. By maintaining fish at 35-39°C instead of 28°C, gene expression can be activated.

Gal4/UAS

One of the most widely used systems is the Gal4/UAS transactivation system, which was adapted from *Drosophila* to the

zebrafish. The system requires the combination of two separate transgenic lines – one expressing the Gal4 protein in a specific cell type and the other containing a construct in which a reporter (e.g. fluorescent protein) or a gene of interest is placed downstream of the UAS sequence, to which the Gal4 protein binds. When these lines are crossed, offspring are generated in which the UAS-driven gene is expressed in those cells expressing Gal4 (reviewed in greater detail by Halpern et al., 2008). The Gal4 system can also be used in an inducible manner, by fusing Gal4 to an ER^{T2} domain, so that Gal4 is expressed upon the administration of tamoxifen. This approach has been useful to study hair cell regeneration (Pinto-Teixeira et al., 2015). However, the Gal4/UAS system in zebrafish is prone to transcriptional silencing and accumulation of methylation (Akitake et al., 2011) over time, thus precluding its use in most cases to study regeneration in adults.

Cre/Lox

A further genetic tool for spatially controlled gene expression is the Cre/Lox recombination system (Felker and Mosimann, 2016; Hans et al., 2009; Langenau et al., 2005). It can also be used for lineage tracing, to assess the origin and fate of cell types during organ regeneration (Kikuchi et al., 2010). A number of systems allow temporal control – most commonly, through fusion of Cre to ER^{T2} (Feil et al., 1997) or by placing Cre under the control of the hsp70 promoter for heat-shock control (Hesselson et al., 2009). This system has been used for temporal gene expression control during the analysis of adult heart regeneration (González-Rosa et al., 2018). It is also possible to use Cre-based approaches to achieve both spatial and temporal control (Kirchgeorg et al., 2018).

TetOn/TetOff

This system allows temporally defined tissue-specific gene expression (Wehner et al., 2015). Its main advantage is that gene expression can be turned on and off by administration or removal of doxycycline – i.e. highly controlled transient expression is possible. It has been used to study Wnt signaling during fin and spinal cord regeneration (Wehner et al., 2014, 2017).

Photoconvertible fluorescent proteins

Finally, photoconvertible Kaede lines have proven useful when the aim is to trace a specific cell or cluster of cells during regeneration. This fluorescent protein undergoes conversion, from green to red light emission, when irradiated under a specific wave-length (Ando et al., 2002). This method has been used for *in vivo* tracing studies of cardiomyocytes during regeneration (Itou et al., 2012). Other photoconvertible proteins are also available.

Gene knock-down/knockout tools

For many decades, the analysis of gene function in the zebrafish relied on non-directed mutagenesis approaches followed by mapping of the mutation. This was followed by the use of transgenic gain-of-function studies or the use of dominant-negative forms or morpholino-based knockdown approaches. Morpholinos are traditionally injected at the 1-cell stage and so cannot easily be used to study regeneration. However, *vivo*-Morpholinos have been developed (by GeneTools) that can be injected into the blood stream or electroporated into a specific tissue and pass through cell membranes. However, there are only few reports on their use and even fewer in the context of regeneration (Bednarek et al., 2015; Hyde et al., 2012; Kizil and Brand, 2011; Thummel et al., 2006). A better approach to understand the effect of the downregulation of a specific gene relies on the use of precise targeted gene editing tools such as TALENs and CRISPR/Cas9. A better approach to understanding the effect of the downregulation of a specific gene relies on the use of precise targeted gene editing tools such as TALENs and CRISPR/Cas9. These have proven useful for generating null mutants in zebrafish (Hwang et al., 2013; Jao et al., 2013) and for assessing gene function during regeneration (Dupret et al., 2017; Dong et al., 2019; Nauroy et al., 2019; Unal Eroglu et al., 2018). The generation of conditional knockouts has also recently been achieved by the directed insertion of loxP sites using CRISPR/Cas9 (Burg et al., 2018). This new approach will be extremely valuable to address gene function during regeneration. Several publications compare the use of all these genetic engineering methods and can provide researchers with the necessary support to choose the best method for their studies (Chatterjee and Lufkin, 2011; Rafferty and Quinn, 2018). Although not yet well established, the possibility of using modified Cas9 versions for transcriptional modulation or induction of epigenetic modifications is also being explored in the zebrafish (e.g. Liu et al., 2019; Long et al., 2015).

Importantly, several screening studies, such as those from the Kawakami (Kawakami et al., 2010) and Brand (Jungke et al., 2013) labs, have led to the generation of large databases with numerous fish lines expressing Gal4 or Cre under specific promoters, which are available to the whole community. These approaches have been recently reviewed (Albadri et al., 2017; Carney and Mosimann, 2018). Similarly, large open source repositories for zebrafish mutants are available (Table 1).

Omics approaches

The different 'omics tools' that have become available in recent years are an important asset for helping researchers gain a deeper knowledge into the mechanisms regulating regeneration. Regarding genomic tools, ChIPseq (Kang et al., 2016; Xiao et al., 2016), Tomo-Seq (Wu et al., 2016), single cell RNAseq (Honkoop et al., 2018 preprint) and histone profiling (Goldman et al., 2017) have been successfully applied to study regeneration in the zebrafish. RNAseq data have provided information, for example, on genes involved in axonal regrowth following spinal cord injury (Mokalled et al., 2016). Complementing these approaches, proteomics (Saxena et al., 2012) and metabolomics (Rabinowitz et al., 2017) are now being used in the context of zebrafish organ regeneration. The possibility of sequencing-based fate mapping will significantly contribute to the study of organ regeneration (Spanjaard et al., 2018). Among the current limitations are the amount of tissue that can be selected for these approaches, annotations of the zebrafish genome and bioinformatics analysis platforms mainly oriented to mammalian models.

Live imaging

One of the main advantages of the zebrafish is the possibility of imaging morphogenetic processes. During embryogenesis, the use of fluorescent reporter lines facilitates imaging of tissues and organs at the cellular and subcellular level. Microscopic techniques such as light sheet microscopy allow for prolonged imaging with minimal interference of physiological processes that occur during either regeneration or development (Ding et al., 2018; Power and Huisken, 2017). The zebrafish larva is also sufficiently transparent that live imaging can be used to follow regenerative processes in organs and tissues in up to 5-day-old larvae or beyond, depending on the organ. The preferred injury model during these stages is genetic ablation, being particularly well suited given the small size of the animals at this age. Automated systems for larval imaging are being developed that facilitate the acquisition and analysis of data, and allow high-throughput screening assays (Early et al., 2018; Pandey et al., 2019).

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In the case of adult zebrafish, *in vivo* imaging of regenerating internal organs can prove more difficult. Nevertheless, short- and long-term imaging of regeneration of external structures have been achieved using fluorescent light microscopy, as described for caudal fin neoangiogenesis (Xu et al., 2015), regeneration of the skin epidermis (Chen et al., 2016) or scale regeneration (Cox et al., 2018). Although more challenging, even internal structures can be imaged and followed through time, as has been shown for adult neural stem cells in homeostasis and during regeneration (Barbosa et al., 2015; Dray et al., 2015). New fish transgenic lines have been developed in the hope of improving *in vivo* imaging of regenerative processes in the adult. Luciferase-based transgenic lines have proven useful for regeneration studies, particularly when looking at internal regenerating structures, as luciferase bioluminescence can be detected even in deep tissues (Chen et al., 2013).

Ultrasound or magnetic resonance imaging have also been used for studying functional recovery after lesion-induced damage (Goessling et al., 2007; González-Rosa et al., 2014; Hein et al., 2015; Koth et al., 2017).

Small molecules and drug screening

Zebrafish are also particularly appropriate for small-molecule and drug-screening studies (Mathew et al., 2007; Matsuda et al., 2018). In this regard, approaches using genetic ablation injuries in larvae are most valuable, as a large number of animals can be screened simultaneously. By using injured zebrafish larvae, multiple compounds can be tested at the same time and used to identify those with a pro-regenerative effect and those that would work to impair regeneration. Screens have identified small molecules with the potential to induce β-cell proliferation and insulin production (Matsuda et al., 2018; Tsuji et al., 2014), and have led to the identification of vitamin D as a potent inducer of cardiomyocyte proliferation in the context of heart regeneration (Han et al., 2019). The use of reporter lines to visualize cell cycle, such as the FUCCI lines (Sugiyama et al., 2009), has proven to be very useful in this regard as it allows one to assess cell proliferation by fluorescent protein expression (Han et al., 2019). The establishment of luciferase promotor lines might also be interesting for screening, in particular for internal structures (Chen et al., 2013).

Cellular and molecular mechanisms of organ regeneration in the zebrafish

So, what has been learned about organ regeneration in the zebrafish using all these tools? The following section aims to provide an overview of different sources of progenitor cells that contribute to rebuild a tissue or organ in this species, and describe some of the common regulatory mechanisms triggering this process.

Cellular origin

Depending on the tissue, and the degree or nature of damage involved, newly generated cells can originate from a number of potential sources (Fig. 3).

Proliferation of existing cell types

In some cases, cells not damaged by the injury can regenerate lost tissue through proliferation – this may involve a partial de-differentiation such that the cells enter a proliferative state, followed by re-differentiation down the same lineage. This is thought to apply to heart regeneration, where pre-existing cardiomyocytes de-differentiate and proliferate (Jopling et al., 2010; Kikuchi et al., 2010) (Fig. 3Ai). Indeed, genetic lineage tracing using a cardiomyocyte-specific promotor showed that all newly formed cardiomyocytes at the site of injury are derived from pre-existent myocardium. Regeneration can also proceed by proliferation of pre-existing cells, without the need for a dedifferentiation step before the proliferation phase. This has been described for liver regeneration – at least when the damage is not too severe and appears similar to the situation in mammals (Michalopoulos, 2007), where regeneration proceeds via proliferation of pre-existing hepatocytes (Sadler et al., 2007) (Fig. 3Aii).

Blastema formation

The replacement of a large area of tissue can occur through the formation of a blastema comprising undifferentiated cells, followed by cell proliferation and differentiation. A good illustration of this process is the regeneration of the zebrafish caudal fin. Upon amputation, an epidermal cap forms that covers the injury. Next, cells from the amputation plane dedifferentiate forming a blastema under the apical epidermal cap. During fin regeneration, the dedifferentiated cells proliferate and undergo re-differentiation to regenerate the various different cell types that make up the fin (reviewed by Pfefferli and Jaźwińska, 2015). The dedifferentiation and proliferation of osteoblasts close to the amputated area make up the new rays (Stewart and Stankunas, 2012) (Fig. 3B). However, it has also been shown that fin regeneration remains viable in the absence of osteoblasts. In this case, new bone cells can originate from a reserve population of osteoblast precursor cells (Ando et al., 2017; Singh et al., 2012).

Transdifferentiation

In some cases, regeneration proceeds via dedifferentiation and proliferation followed by re-differentiation into a different mature cell type from the starting population. One example of this is the process following severe hepatocyte loss in the liver. Under these circumstances, regeneration proceeds initially via the proliferation of biliary epithelial cells (Fig. 3C), which differentiate into hepatocytes (Choi et al., 2014). Transdifferentiation has also been observed in zebrafish pancreatic regeneration. A study searching for genes that would promote β-cell regeneration identified a protein, Igfbp1, that potentiated β-cell regeneration by stimulating α- to β-cell transdifferentiation (Lu et al., 2016).

Differentiation of tissue-resident stem cells/progenitors

Not all organs regenerate through dedifferentiation and proliferation of mature cells. Injuries to kidney, skeletal muscle or neural tissues resolve by the proliferation and differentiation of stem cells. For example, kidney regeneration following chemical damage proceeds via the proliferation of adult nephron progenitor cells (Diep et al., 2011). As described in mammals (Wosczyna and Rando, 2018; Young and John, 2000), skeletal muscle regeneration in zebrafish requires the proliferation of satellite cells, a population of adult $Pax7^{+}$ muscle stem cells that, after an injury to the muscle, proliferate and fuse with adjacent muscle fibers (Berberoglu et al., 2017). Regeneration of neurons also has been shown to depend on a specific progenitor cell population. In the injured zebrafish telencephalon, new neurons are derived from a subpopulation of radial glial cells (Kroehne et al., 2011) that have been demonstrated to function as a stem cells population in uninjured brain tissue (Rothenaigner et al., 2011) (Fig. 3D).

Environmental cues driving regeneration

Initial wound closure is followed by an inflammatory response mediated by neutrophils and macrophages, which are attracted by

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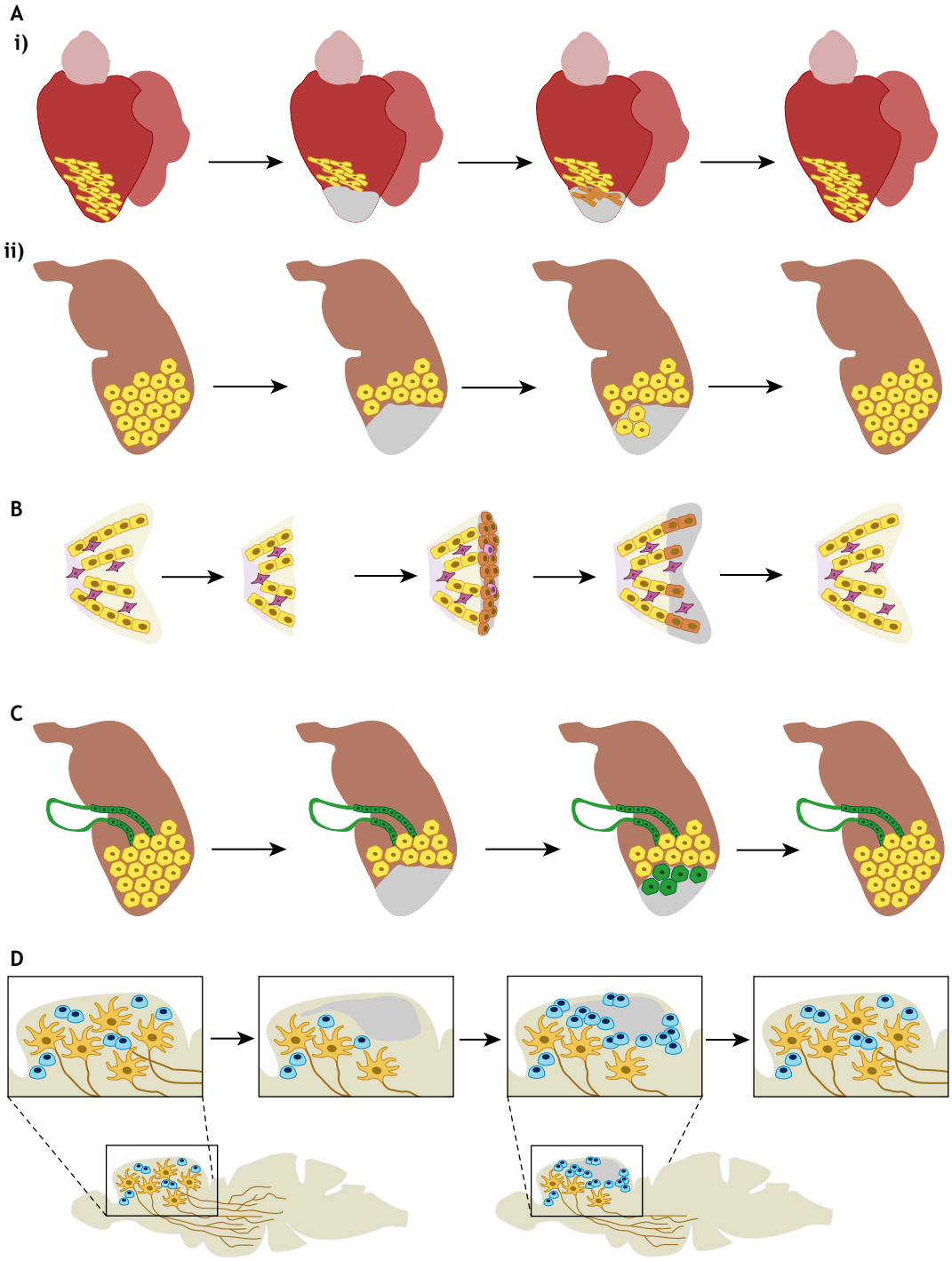

Fig. 3. Cellular origin of the *de novo* formed tissue during organ regeneration in the zebrafish. (A) Dedifferentiation, proliferation and re-differentiation. (Ai) In the heart, cardiomyocytes in close proximity to the injury revert to a less differentiated stage, re-enter the cell cycle and redifferentiate into mature cardiomyocytes. (Aii) During regeneration of minor liver damage, hepatocyte regeneration occurs with no signs of dedifferentiation prior to cell cycle entry and proliferation. (B) Blastema formation as an intermediate step during regeneration. After fin amputation, cells of various lineages – including osteoblasts – dedifferentiate and accumulate under an apical epidermal cap. They then proliferate and redifferentiate to rebuild the missing fin structures. (C) Phenotypic switch or transdifferentiation during regeneration. Example: after extensive liver damage, biliary ductal cells (green) can transdifferentiate into hepatocytes (green hexagonal cells) that then differentiate into mature proliferating hepatocytes. (D) Stem cells as progenitor cells. Neural stem cells/progenitor cells proliferate and differentiate into new neurons during regeneration of the central nervous system. While neuronal regeneration has been well described, less information is available on robust axon regrowth. Yellow, differentiated cells; orange, dedifferentiated cells; purple, non-osteoblast cells within the fin; green hexagonal cells, cells undergoing transdifferentiation; blue, stem cells/progenitor cells. Damaged area is shown in gray.

signals generated from the dying cells and surrounding tissue, and home to the site of damage. Indeed, inflammation is essential for organ regeneration (Mescher, 2017). An inflammatory response precedes zebrafish brain regeneration (Kyritsis et al., 2012) and has been suggested to be important for heart regeneration (Lai et al., 2017). Furthermore, T lymphocytes are involved in the regeneration of several

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organs, such as spinal cord, heart and retina (Hui et al., 2017). Supporting these findings, genetic or chemical ablation of phagocytic macrophages during regeneration results in impaired fin (Li et al., 2012; Petrie et al., 2014), lateral line (Carrillo et al., 2016), heart (Lai et al., 2017) and spinal cord (Tsarouchas et al., 2018) regeneration.

The ECM and the stromal environment also influence regeneration. Not only do they provide structural support for progenitor cells, but they also have an impact on their proliferation and differentiation. Indeed, fibrosis and regeneration are tightly linked processes. For example, the pro-fibrotic molecule TGFβ induces not only fibrosis but also cardiomyocyte proliferation (Chablais and Jazwinska, 2012). Moreover, ablation of collagen-producing cells impairs cardiomyocyte proliferation after heart injury (Sánchez-Iranzo et al., 2018), and ECM proteins are required for spinal cord regeneration (Mokalled et al., 2016; Wehner et al., 2017).

The nervous system is also key for adequate regeneration. This has been demonstrated both in the fin, where denervation following amputation results in impaired regeneration (Simões et al., 2014), and in the heart, where regeneration is also impaired following inhibition of peripheral nervous system activity (Mahmoud et al., 2015). Future work is needed to further unravel the role of the nervous system during organ regeneration, which can range from secretion of molecules to establishment of synaptic connections.

Finally, revascularization is also essential for proper regeneration. When this process is impaired during cardiac injury, regeneration is blocked. Studies conducted on this subject suggest that revascularization of the injured area is essential to stimulate cardiomyocyte proliferation (Harrison et al., 2015; Marín-Juez et al., 2016). The exact mechanisms through which revascularization contributes to regeneration remain to be discovered.

Molecular pathways promoting regeneration

The key signaling pathways involved in embryonic development are often also involved in organ regeneration. To study their function, researchers have used transgenic lines for temporal and tissue-specific overexpression of a core gene of a particular pathway and studied the effect on organ regeneration. In addition, analysis of organ regeneration in mutants of a gene involved in the pathway of interest has been informative. Here, we provide a few examples of how such pathways contribute to regenerative processes in zebrafish; we refer the reader to the papers cited for further details. One example of a pathway important for regeneration is the retinoic acid (RA) pathway. RA regulates FGF, Wnt/β-catenin and Igf signaling during blastema formation in the regenerating caudal fin (Blum and Begemann, 2012), and stimulates cardiomyocyte proliferation, likely in a paracrine manner (Kikuchi et al., 2011). The fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) pathways, which are essential for organ growth and differentiation (Kan et al., 2009; Smith et al., 2006), are also involved in regeneration. BMP signaling has been implicated in the regeneration of several organs, including the liver, the heart and the fin (Kan et al., 2009; Sehring et al., 2016). Similarly, FGF is involved in multiple processes of regeneration, including the control of blastema proliferation during fin regeneration (Lee et al., 2005; Poss et al., 2000) or heart regeneration (Lepilina et al., 2006). One of the most universally used pathways in tissue regeneration throughout the animal kingdom is the Wnt signaling pathway (Slack, 2017). In the zebrafish, it regulates blastema proliferation in amputated caudal fin among many other processes (Stoick-Cooper et al., 2007; Wehner et al., 2017). Juxtacrine signaling pathways, such as the Notch pathway are also associated with proliferation control both in fin (Münch et al., 2013) and heart (Zhao et al., 2014). Other pathways involved in the control of cell cycle include the Hippo-Yap/TAZ pathway (Flinn et al., 2018; Mateus et al., 2015), which has recently received considerable attention in the field of regeneration because it links the effect of a changing environment and mechanical forces in response to injury with the regenerative response in the surrounding cells. In all cases, specificity and temporal control of these pathways is crucial for promoting regeneration. In addition, depending on the combination of active pathways, different cellular responses are elicited, ranging from cell differentiation or proliferation to migration to epithelial-to mesenchymal transition, all of which are crucial for organ regeneration.

In sum, during zebrafish regeneration, cells that repopulate injured areas can have different origins, depending not only on the lesioned organ, but also on the extent and the type of injury. Furthermore, for regeneration to succeed there must be a balance between the signals that induce cell proliferation and those that halt cell division and determine cell fate.

Extent and limits of the regenerative capacity in zebrafish

Without doubt, the zebrafish is an organism with an amazing capacity for regeneration. Here, we discuss some of the mechanisms that might underlie this capacity, but also highlight some possible limitations of organ regeneration in this animal.

Possible explanations for the high regenerative capacity

The regenerative capacity in the zebrafish might rely on its capacity to activate specific gene regulatory networks in response to injury that are repressed in other animal models (Yang and Kang, 2018). Indeed, epigenetic modifications are necessary to trigger regeneration. One of the earliest findings in this field reported that, in the fin, histone modifications precede initiation of regeneration (Stewart et al., 2009), and histone methyltransferases are necessary for this process (Dupret et al., 2017). The use of ChIPseq and transgenesis has provided much insight into the landscape of active enhancers during organ regeneration, identifying enhancer elements that are activated in response to injury consistently in different organs and even in different organisms (Kang et al., 2016; Pfefferli and Jaźwińska, 2017).

One important characteristic of zebrafish is its capacity to grow throughout its lifespan, meaning that most of the zebrafish cells, e.g. cardiomyocytes, retain their proliferative capacity (Wills et al., 2008). For heart regeneration, cardiomyocyte ploidy has been shown to be a limiting step. Zebrafish cardiomyocytes are mononuclear and diploid, whereas in adult humans they are mostly polyploid. Indeed, induction of polyploidy in zebrafish cardiomyocytes abrogates heart regeneration (González-Rosa et al., 2018; Patterson et al., 2017).

The immune system might play a key role in defining the regenerative capacity of an organism. As an example, differences in immune response to cardiac injury in two teleost models (medaka and zebrafish) correlate with reduced regenerative capacity of medaka (Lai et al., 2017). Finally, regenerative capacities might also be attributed to differences in the environment that species inhabit – exemplified by the differences in heart regeneration in surface versus cave morphs of the cavefish *Astyanax mexicanus* (Stockdale et al., 2018). Thus, using other fish models with different tissue recovery capacities for regeneration studies (see Table 4) may contribute to a better understanding of the different mechanisms underlying this process.

Unlimited regeneration in the zebrafish?

Regeneration in the zebrafish seems to be nearly unlimited: regeneration of the caudal fin and barbell occurs even after

Table 4. Other teleost fish models of regeneration

Fish model	Organs in which regeneration has been studied
Astyanax mexicanus (Mexican cave fish)	Heart (Stockdale et al., 2018)
Carassius auratus (Goldfish)	Spinal cord (Bernstein and Gelderd, 1970) Eye (Parrilla et al., 2012) Fin (Caskey and O'Brien, 1948) Heart (Grivas et al., 2014)
Oryzias latipes (Medaka)	Kidney (Watanabe et al., 2009) Fin (Nemoto et al., 2007) Pancreatic β-cells (Otsuka and Takeda, 2017)

repetitive amputations (Azevedo et al., 2011; LeClair and Topczewski, 2010), although subtle changes to the newly formed organ, such as variation in the pigmentation patterning of the fin or the position of the bony ray bifurcations, have been observed (Azevedo et al., 2012). Moreover, age does not seem to limit regenerative capacity, at least for the heart (Itou et al., 2012). Nevertheless, there is evidence that organ regeneration can be partially incomplete. For example, although heart morphology is restored after lesion and no scar is visible, the ventricular wall motion of the regenerated area remains partially affected and contracts asynchronously with the remainder of the heart, even after complete tissue regeneration (González-Rosa et al., 2014). Furthermore, following resorption of extracellular fibrotic components during heart healing, accumulated fibroblasts are not fully eliminated after complete regeneration but become inactivated (Sánchez-Iranzo et al., 2018). It is thus important to distinguish between morphological and functional regeneration and to recognize that – even in a system such as zebrafish – regeneration may not be functionally 'perfect'.

Conclusions and perspectives

In conclusion, using the zebrafish as an animal model in regeneration studies has undoubtedly contributed to the development of the field but, as with any other model, there are advantages and disadvantages to consider (Table 5). Despite the contributions made, there are still unanswered questions. For example, there is little information on how metabolism is linked to regenerative capacity. Furthermore, the interplay between fibrosis and regeneration, as well as the mechanisms of fibrotic tissue regression, are still not fully understood. Why is a scar formed under some circumstances or in some organs while in other cases regeneration occurs? To what extent does regeneration rely on a transient fibrotic scar? It is also important to understand how mechanical forces influence regeneration – studies in the zebrafish might help to explain how, for example, stiffness or shear stress regulates organ regeneration. In addition, it is intriguing that regenerating tissues stop growing at the appropriate size; this is poorly understood. Indeed, there is still a dearth of knowledge regarding which cell populations become active during regeneration and whether all the cells have the same regenerative potential. To answer many of these questions, some of the molecular tools such as conditional genome editing will need to be improved and widely established for regeneration studies. Advances in intra vital imaging for adult zebrafish will also help to expand our toolbox. With these new technological advances in hand, important discoveries on the mechanisms of organ regeneration using the zebrafish model will certainly be achieved. A deep knowledge on the mechanisms of organ regeneration in species such as the zebrafish with innate regenerative capacity might serve as inspiration for the development of therapeutic strategies in mammals.

Table 5. Advantages and disadvantages of the zebrafish as a regeneration model

Advantages	Disadvantages
High regenerative capacity	Non-mammalian model
Vertebrate model	Partial genome duplication
Established husbandry protocols	Dearth of antibodies for protein studies
Low-cost maintenance	Difficulty in performing *in vivo* imaging in adult stages
External development	Difficulty in using some molecular tools (e.g. knock-ins)
Possibility of working with large numbers	Anatomical and physiological differences to humans (e.g. two-chambered heart)
Ease of *in vivo* imaging during development	
Genetic engineering, annotated genome	
Physiological similarities with humans (e.g. heart rate)	

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Competing interests

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